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Possible involvement of radical reactions in desialylation of LDL

Kyoko Tanaka[a], Sadako Tokumaru[b], Shosuke Kojo[a,*]

[a] *Department of Food Science and Nutrition, Nara Women's University, Nara 630, Japan*
[b] *Department of Life and Health Sciences, Joetsu University of Education, Joetsu, Niigata 943, Japan*

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Abstract The role of oxidatively modified LDL in the pathogenesis of atherosclerosis has been well documented. These studies have focused on modifications of lipid and protein parts of LDL. Recently desialylated LDL has received attention in relation to atherosclerosis and coronary artery disease. We examined the possible involvement of radical reactions in desialylation of LDL. Human LDL was subjected to oxidative damage using Cu^{2+} ion. As the conjugated dienes monitored by absorption at 234 nm increased, the content of sialic acid decreased steadily. Both the elevation of conjugated diene and the decrease of sialic acid were inhibited by β-mercaptoethanol, a typical radical scavenger. Besides, both butylated hydroxytoluene and a nitrogen atmosphere inhibited the decrease of sialic acid. These inhibition experiments suggested that sialic acid moieties in LDL were reactive toward radicals.

Key words: Low density lipoprotein; LDL; Sialic acid; Desialylation; Lipid peroxidation

1. Introduction

The role of oxidized low density lipoprotein (LDL) in atherogenesis has been well established [1–3]. Studies on the oxidation of LDL have been focused mainly on lipid peroxidation and resulting modification of apolipoprotein B by aldehydes [4,5]. Recently it was found that the content of desialylated LDL in atherosclerotic patients was about 3-fold higher than that in healthy subjects [6] and that desialylated LDL induced cholesterol [6–8] and lipid [8] accumulation in smooth muscle cells. Besides desialylation caused retardation of catabolism of LDL in diabetics [9]. These studies demonstrated the presence of naturally desialylated LDL in plasma but the mechanism of desialylation as well as the structure of desialylated LDL remained to be explored. We report here that radical reactions are a possible mechanism of desialylation of LDL.

2. Materials and methods

2.1. Reagents

NANA (sialic acid) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). 1,2-Diamino-4,5-methylenedioxy-benzene dihydrochloride (DMB) was obtained from Dojindo Laboratories (Kumamoto, Japan). All other reagents were of analytical grade and were purchased from Wako Pure Chem. Co. Ltd. (Osaka, Japan).

*Corresponding author. Fax: (81) (742) 203459.

Abbreviations: BHT, butylated hydroxytoluene; DMB, 1,2-diamino-4,5-methylenedioxy-benzene dihydrochloride; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; NANA, *N*-acetylneuraminic acid (sialic acid)

2.2. LDL isolation

Blood was taken from healthy volunteers after over night fasting and the serum was separated. After the addition of 5% EDTA solution at pH 7.4 to the final concentration of 0.1%, LDL was prepared by ultracentrifugation according to the method of Hatch and Lees [10].

2.3. Oxidation of LDL and determination of conjugated diene

Oxidation of LDL was carried out as described in [11]. Removal of EDTA and salt of the density gradient from the LDL solution was conducted with a prepacked column (Econo-Pac 10DG, Bio-Rad, Richmond, CA, USA) as described in [11]. EDTA-free LDL solution (50 μg protein/ml PBS) was transferred into a brown-colored tube with a Teflon covered screw cap and the oxidation was started at 37°C by the addition of 0.1 mM aqueous $CuSO_4$ (1/60 volume of LDL solution) to a final concentration of 1.67 μM [11]. Conjugated diene was determined based on the absorption at 234 nm [11].

2.4. Measurement of NANA

NANA concentration was determined based on the method of Hara et al. [12]. A 100 μl aliquot was taken from the reaction mixture into a microtube (1.5 ml volume) and 5 μl of 500 mM H_2SO_4 and 5 μl of 4 mM EDTA were added. The mixture was hydrolyzed at 80°C for 1 h. The reaction time was determined by separate experiments using LDL to give a plateau value. After cooling in ice, 100 μl of 7 mM of DMB aqueous solution containing 1.0 M β-mercaptoethanol and 18 mM sodium hydrosulfite was added and the mixture was incubated at 60°C for 2.5 h. The reaction mixture (10 μl) was directly subjected to HPLC analysis. The DMB derivatives of NANA were separated on a reversed phase column (Radial-Pak C18 cartridge (100×8 mm i.d.; particle size 5 μm) Waters, Milford, MA, USA) using a mixture of methanol-acetonitrile-water (25:4:91, v/v) as a mobile phase at a flow rate of 1.2 ml/min [12]. Detection was by a fluorescence detector (type RF-535, manufactured by Shimadzu Co. Ltd., Kyoto, Japan) using excitation at 373 nm and emission at 448 nm [12]. As a calibration, 100 μl of standard NANA solution (0, 1, and 5 μM) was treated similarly. The levels of NANA and conjugated diene were expressed as mean ± S.D. for three independent runs and analyzed by ANOVA using StatView software (Abacus Concepts, Berkeley, CA). Differences between group means were analyzed using Bonferroni/Dunn (Dunn's procedure as a multiple comparison procedure) generated by this program. Differences were considered significant at $P < 0.05$.

2.5. Inhibition experiments

For inhibition experiments, β-mercaptoethanol (final concentration at 0.1 or 2 mM) or butylated hydroxytoluene (BHT: final concentration at 0.1 or 1 mM) was added to 1 ml of LDL solution (50 μg/ml PBS) and then 16.7 μl of $CuSO_4$ solution (0.1 mM) was added to initiate the reaction at 37°C. BHT was added as 10 μl of methanol solution. At the start and after 6 h, contents of conjugated diene and NANA were determined as described above. For inhibition experiments by nitrogen, nitrogen gas was gently introduced on the surface of LDL solution (50 μg/ml PBS) for 3 min at 0°C and then 16.7 μl of $CuSO_4$ solution (0.1 mM) was added to initiate the reaction at 37°C.

2.6. Protein assay

Protein concentrations were determined according to the method of Lowry et al. [13] using bovine serum albumin as the standard.

3. Results and discussion

Human LDL was subjected to a well-studied oxidation [11]

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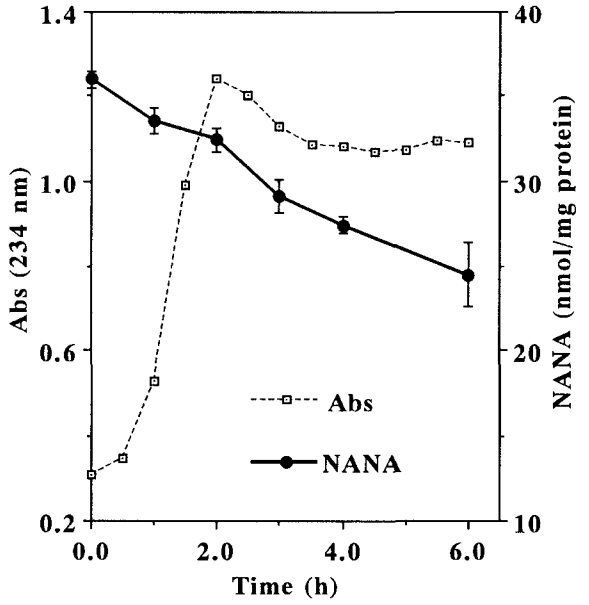

Fig. 1. Changes of conjugated diene and NANA during oxidation of LDL with Cu^{2+}. LDL was subjected to oxidation initiated by Cu^{2+} ion at 37°C. The absorption at 234 nm (□) (conjugated diene) and NANA (●) were determined as described in Section 2. Each point was mean ± S.D. of three independent runs. The bars are the standard deviations. Where no bars are shown, S.D. was smaller than the symbol.

initiated by Cu^{2+} ion at 37°C. The extent of lipid peroxidation was monitored based on conjugated diene (Fig. 1), which showed a typical time course of these reactions as described [11]. Each point in Fig. 1 was mean ± S.D. of three independent runs. At 1 h and thereafter, the level of conjugated diene was significantly higher than that of the start.

At the same time, NANA content was also determined. In previous works [6–9,14], NANA was determined by the conventional colorimetric method [15] involving oxidation of NANA into malondialdehyde and its quantitation using the reaction with thiobarbituric acid. However malondialdehyde and thiobarbituric acid reactive substances (TBARS) are well known products of lipid peroxidation. Therefore the method [15] may give an inaccurate result when it is applied to analyze changes in NANA in a process involving lipid peroxidation. In the present work, a method [12] involving chemical conversion and HPLC was used to quantitate NANA in a specific manner, because specific determination of a key molecule is a prerequisite to investigate radical reactions in biology systematically, as in our previous studies [16–22].

The content of NANA in LDL decreased with the progression of lipid peroxidation. In Fig. 1, a typical example is shown. At 2 h and thereafter, the content of NANA decreased significantly compared to the starting value (35.96 ± 0.48 nmol/mg protein). The initial content of NANA depended on LDL preparations but the result that conjugated diene increased and NANA decreased on oxidation with Cu^{2+} was always obtained. It was confirmed that direct addition of Cu^{2+} to free NANA did not cause degradation of NANA. These results suggested that NANA in LDL was converted to unknown compound(s) during lipid peroxidation and this reaction was not a simple hydrolysis of the glycosyl bond of NANA. To investigate the relationship between the decrease of NANA and radical reaction, an inhibition study utilizing β-mercaptoethanol was made. At the start, absorption at 234 nm of the control LDL solution was 0.226 ± 0.002. After 6 h of the oxidation reaction with Cu^{2+}, conjugated diene increased to an absorption of 0.964 ± 0.033. When the reaction was made simultaneously using the same batch of LDL in the presence of β-mercaptoethanol at 2 mM, the absorption at 234 nm after 6 h was 0.533 ± 0.021, which was significantly lower than the control run (without the radical scavenger). NANA content at the start was 33.23 ± 0.69 nmol/mg protein, which decreased to 26.75 ± 0.57 nmol/mg protein after the control reaction for 6 h. In the presence of β-mercaptoethanol at 2 mM, the level of NANA after the reaction for 6 h was 32.51 ± 0.28 nmol/mg protein, which was significantly higher than the control experiment which was performed in the absence of the radical quencher. Significant inhibitory effects on the formation of conjugated dienes and desialylation caused by Cu^{2+} were observed when a lower concentration (100 μM) of β-mercaptoethanol was used.

Decrease of sialic acid in LDL by Cu^{2+} was significantly inhibited by BHT at 1 mM (the change by Cu^{2+} was from 33.0 ± 0.48 to 20.66 ± 1.73 nmol/mg protein whereas that in the presence of BHT was to 26.89 ± 2.28). A significant inhibition by BHT was also observed when BHT was used at the concentration of 100 μM (the change by Cu^{2+} was from 27.89 ± 1.00 to 20.07 ± 0.26 while that in the presence of BHT was to 23.21 ± 0.84 nmol/mg protein). To exclude the effect of methanol, a solvent for BHT, the reaction was made in the presence of the same concentration of methanol with the run in the presence of BHT. In the reaction, NANA decreased to 21.32 ± 0.47 nmol/mg protein, which was not significantly different from the reaction by only Cu^{2+} but significantly different from that in the presence of BHT at 100 μM. This result excluded the effect of methanol. The formation of conjugated dienes was also inhibited significantly by BHT at 100 μM (the change by Cu^{2+} was from 0.228 ± 0.001 to 0.880 ± 0.044 while that in the presence of BHT was to 0.639 ± 0.114). The effect of methanol on the formation of conjugated dienes was not also observed. When the reaction was made for 6 h under reduced oxygen pressure by replacing the air in the reaction vial with a gentle stream of nitrogen gas, the decrease of sialic acid was inhibited significantly (the change by Cu^{2+} was from 33.23 ± 0.69 to 26.75 ± 0.57 while that under nitrogen was to 28.33 ± 0.17 nmol/mg protein). These inhibition experiments suggested a close relationship between radical reactions and the disappearance of NANA. The structure of the product(s) from NANA in the oxidation of LDL remained to be explored.

These results supported the idea that NANA attached on LDL presumably on apoB was reactive toward radicals capable of antioxidant behavior. This observation was consistent with the result [23] that addition of NANA increased the lag phase in the oxidation of lipoprotein (a) treated with neuraminidase, although NANA did not affect the oxidation of LDL [23]. The labile character of NANA among sugars toward hydrogen atom abstraction by electrophilic oxygen radicals may be expected based on its structure, i.e., having secondary C–H bonds, whose carbon atom does not bear electron-withdrawing hydroxyl group and has electron-rich carboxylate in the neighborhood. Thus it may be important to investigate the role of NANA as a regulator of radical

reaction leading to catabolism of glycoproteins which have NANA moiety on the surface where activated oxygen species are assumed to attack at first.

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